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PUTATIVE NITROXIDERGIC CELLS IN THE DIGESTIVE SYSTEM OF SOME MYTILIDS (MOLLUSCA: BIVALVIA: MYTILIDAE) REVEALED BY NADPH-DIAPHORASE HISTOCHEMISTRY

Eugenia A. Pimenova & Anatoly A. Varaksin

Institute of Marine Biology, Russian Academy of Sciences, Vladivostok 690041, Russia; eapimenova@yandex.ru

ABSTRACT

Distribution and morphology of putative nitroxidergic (NO-ergic) cells were studied in the labial palps, esophagus, stomach, digestive gland, and intestine of the bivalves *Crenomytilus grayanus*, *Modiolus modiolus*, and *Mytilus coruscus* using NADPH-histochemistry (Hope & Vincent, 1989). NO-producing elements were found in all examined organs and regions of the digestive system. NADPH-diaphorase-(d)-positive staining was readily detectable in neurons, secretory cells, brush border epithelial cells, and in the nervous plexuses. Intraepithelial nerve cells were the most common NADPH-d-positive cell type throughout the digestive tube. These cells had a fusiform perikaryon, which gave rise to a thin apical process that extended toward the gut lumen, whereas the basal process contacted the basiepithelial NADPH-d-positive plexus. In the major typhlosole of *C. grayanus*, these cells constituted up to 1.28% of the total number of epithelial cells. The bivalve species studied exhibit a similar distribution pattern of NADPH-d-positive cells, which lie separately or form small groups of two to three in the basal part of the epithelium, being most abundant in the dorsal and ventral regions. Subepithelial NADPH-d-positive neurons were found in *C. grayanus* and *M. modiolus.* The labial palps, lips, and esophagus contained an abundance of intraepithelial and subepithelial secretory cells that stained positive for NADPH-diaphorase. Moreover, NADPH-diaphorase was detected in brush border epithelial cells of the primary and secondary ducts of the digestive gland. All examined regions of the digestive system contained basiepithelial and subepithelial NADPH-d-positive nerve fibers that formed loose to highly developed plexuses. The most prominent plexuses were found in the lips, esophagus, and intestine of the species studied.

Key words: NADPH-diaphorase, nitric oxide (NO) synthase, enteric nervous system, neurons, nervous plexuses, secretory cells, brush border epithelial cells, bivalve.

INTRODUCTION

To date, nitric oxide has been reported to be enzymatically produced within the cell and appears to be involved in the control of cellular and tissue metabolism both in vertebrates and invertebrates. The role of NO in digestive physiology and distribution of NO-producing enzymes in the alimentary canal has been extensively studied in higher (Schleiffer & Raul, 1995; Shah et al., 2004) and lower vertebrates (Olsson & Karila, 1997; Lamanna et al., 1999). On the other hand, our knowledge of the distribution and function of nitric oxide in feeding and digestion of invertebrates is extremely poor. NADPH-diaphorase, an indicator of nitric oxide synthase activity, was detected in the pharynx of the freshwater planarian *Dugesia tigrina* (Esiksson, 1996), trematode *Fasciolopsis buski* (Tandon et al., 2001). Selective histochemical staining was also present in the enteric nervous system of the nematode *Ascaris suum* (Bascal et al., 1995) and the snail *Helix lucorum* (Röszer et al., 2005). NO-ergic cells were also found in the cardiac stomach of asteroids (Martinez et al., 1994; Elphick & Melarange, 1998) and esophagus of gastropods, where NO was shown to act as a sensory neuromodulator (Moroz & Gillette, 1996; Hurst et al., 1999). The role of nitric oxide in the induction and control of feeding response was shown in the *Hydra vulgaris,* which possesses a very primitive olfactory-like system (Colasanti et al., 1997). NO-synthase

activity was detected in the centers that control the feeding rhythms, for example, in the buccal ganglia of *Lymnaea stagnalis* (Kobayashi et al., 2000) and *Clione limacina* (Moroz et al., 2000). Nitric oxide was revealed in the epithelial cell of the salivary glands in the blood-sucking bug *Rhodnius prolixus*, where NO was involved in the control of secretion of nitrophorins (Nussenzveig et al., 1995). Moreover, nitric oxide seems to play an important role in feeding and digestion in bivalves; however, the available information on the distribution and function of NO in bivalves is restricted to our reports (Pimenova et al., 2002; Varaksin et al., 2002; Pimenova & Varaksin, 2003).

The present paper focuses on the distribution and morphology of putative nitroxidergic cells and plexuses in all regions of the digestive system in the bivalve mollusks *Crenomytilus grayanus*, *Modiolus modiolus*, and *Mytilus coruscus* (Mytilidae). Elsewhere, we have also reported that nitroxidergic cells occur in the intestine of *C. grayanus* (Varaksin et al., 2002) and *Modiolus kurilensis* (Pimenova et al., 2002), as well as in the labial palps, lips, and esophagus of *Crenomytilus grayanus* (Pimenova & Varaksin, 2003). Our paper combines these scattered data with new original findings to present a general concept of distribution and morphological types of nitroxidergic cells in the digestive system of the most abundant mytilids of the Sea of Japan.

MATERIALS AND METHODS

The study was carried out on mature male and female individuals of the marine bivalves *Crenomytilus grayanus*, *Modiolus modiolus*, and *Mytilus coruscus*, which were collected from Amurskii and Ussuriiskii Bays (Sea of Japan) in the fall 1998–2002. The animals were allowed a two to three days adaptation period, during which they were kept in aquaria with aerated seawater and received no food.

Morphological Methods

For routine morphological examination, portions of the labial palps, lips, esophagus, stomach, digestive gland, and intestine were fixed in Bouin's fluid and/or in 4% paraformaldehyde. Paraffin and/or cryostat (20 µm thick) sections were stained with Ehrlich's hematoxylin and eosin following the generally accepted protocols (Merkulov, 1969).

NADPH-Diaphorase Histochemistry

NO-ergic cells were visualized using NADPH-diaphorase histochemistry (Hope & Vincent, 1989). Positive staining for NADPH-diaphorase has been widely used as a marker for NO-synthase both in vertebrates (Hope et al., 1991; Lamanna et al., 1999) and invertebrates, including mollusks (Moroz & Gillette, 1996; Newcomb & Watson, 2001). The technique is based on the NADPH-dependent conversion of nitroblue tetrazolium, an exogenous substrate, to insoluble diformazan deposits.

Portions of the labial palps, lips, esophagus, stomach, digestive gland, midgut, and hindgut were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4°C. After fixation, the specimens were rinsed in three to four changes of 15% sucrose in 0.15 M Tris-HCl buffer (pH 8.0) for 20 h at the same temperature. Cryostat sections were cut at a thickness of 20 and 40 µm. Complete series of longitudinal and transverse sections were incubated in 0.15 M Tris-HCl buffer (pH 8.0) containing 0.5 µM ß-NADPH (Sigma), 0.5 µM nitroblue tetrazolium (Sigma), and 0.3% triton X-100 for 1 h at 37°C. Then they were rinsed in two changes of distilled water, dehydrated through an ethanol series, and mounted in Dammar Resin. The specificity of the staining was evaluated by adding 10 µM N^{ω}-nitro-L-arginine, and by omitting ß-NADPH or nitroblue tetrazolium.

Statistical Analysis

For each portion of the digestive system, the total number of intraepithelial NADPH-diaphorase-positive cells was counted in ten transverse 20 µm thick sections, and the mean number of cells per section was calculated. For the lips, upper, middle and lower esophagus, as well as for the midgut and hindgut, the number of these cells was expressed as a percentage of the total number of epithelial cells.

Cell width and height, as well as the thickness of the basi- and subepithelial plexuses were measured with an ocular grid and an object micrometer.

The data were processed with Statistica 5.0 software and MS Office package for Windows 2000. The mean and standard deviation was calculated using the unequal variance paired t-test. The difference was considered significant at $p < 0.05$ confidence level.

The slides were examined and photographed with an Olympus BH2-RFCA (model BHS) light microscope.

TABLE 1. Sizes of intraepithelial NADPH-diaphorase-positive secretory cells and nerve plexuses in the labial pulps of bivalve mollusks (Mytilidae); + indicate that loose networks of NADPH-d-positive nerve fibers were found. 0 indicate that NADPH-d-positive elements are absent.

Species	Ridged side			Smooth side		
	cells, μm		plexuses	cells, μm		plexuses
	width	height		width	height	
Crenomytilus grayanus	6.5 ± 0.2	16.3 ± 0.2	0	7.0 ± 0.2	15.4 ± 0.2	0
Modiolus modiolus	0	0	+	6.6 ± 0.1	13.4 ± 0.2	0
Mytilus coruscus	7.1 ± 0.1	17.8 ± 0.1	0	6.1 ± 0.1	9.7 ± 0.2	0

RESULTS

Labial Palps

The bivalve species examined have two pairs of leaf-like labial palps, which lie on either side of the mouth. The oral surfaces of the palps is deeply folded, whereas the outer surfaces are comparatively smooth. The folded structure of the oral surface is due to conspicuous grooves and ridges, the latter leading down to the mouth.

NADPH-diaphorase labeling was detected in numerous secretory cells. The vacuolated cytoplasm was foamy in appearance; the surface contour of the nucleus was often difficult to discern (Fig. 2A). NADPH-d-positive secretory cells occurred on both surfaces of the labial palps in *C. grayanus* and *M. coruscus* and only on the smooth surface in *M. modiolus.* On the smooth side, only intraepithelial cells stained positive for NADPH-diaphorase, while both intraepithelial and subepithelial cells were observed on the ridges side (Fig. 1A). The intraepithelial cells had a large goblet or oval cell body (Table 1) and a distally distended apical process. These cells were often clustered together and occasionally formed large groups, in contrast to the subepithelial cells, which were usually scattered singly. The subepithelial cells were morphologically similar to the intraepithelial cells; the only difference was that the former were more regularly rounded in shape.

The epithelium of the smooth side of the labial palps of *C. grayanus* also contained rare NADPH-d-positive cells with a homogeneously stained cytoplasm and a completely unstained nucleus (Fig. 2A). These cells seemed to be neurons. Their comparatively small subspherical cell bodies gave rise to two thin processes, one of which extended towards the apical surface of the epithelium, whereas another one ran in the opposite direction. On the ridged side of the labial palps, solitary thin subepithelial NADPH-d-positive fibers occurred only in *M. modiolus* (Table 1). They mostly ran parallel to the long axis of the palp and had varicosities along their length. No fibers were observed on the smooth side of the labial palps.

Lips

The ridged surface of the basal part of the labial palps are continuous with smooth regions that give rise to long narrow lips. The latter fuse together thereby surrounding the mouth opening.

In the lips, diformazan deposits labeled two cell types and plexuses. Intra- and subepithelial NADPH-d-positive cells of the first type were found in all the mytilid species examined (Figs. 1A, 2B). They were morphologically similar to NADPH-d-positive secretory cells of the labial palps and were most abundant in the dorsal and ventral regions of the lips. The largest intraepithelial cells, measuring 11.4 ± 0.2 μm in width by 21.6 ± 0.8 μm in height were found in *C. grayanus*.

NADPH-d-positive cells of the second type were most probably neurons. They were found in the lip epithelium of *C. grayanus* and *M. modiolus* (Table 2). These cells had a spindle-shaped cell body, from which an apical process extended toward the apical surface of the epithelium, while the basal one penetrated into the basal portion of the epithelium. The perinuclear cytoplasm and processes showed clear positive labeling, whereas the nucleus was mostly unstained (Fig. 2C). The cells lay in the basal part of the epithelium, being most abundant in the dorsal and ventral regions. They were either scattered singly or clustered together to form groups of two to three.

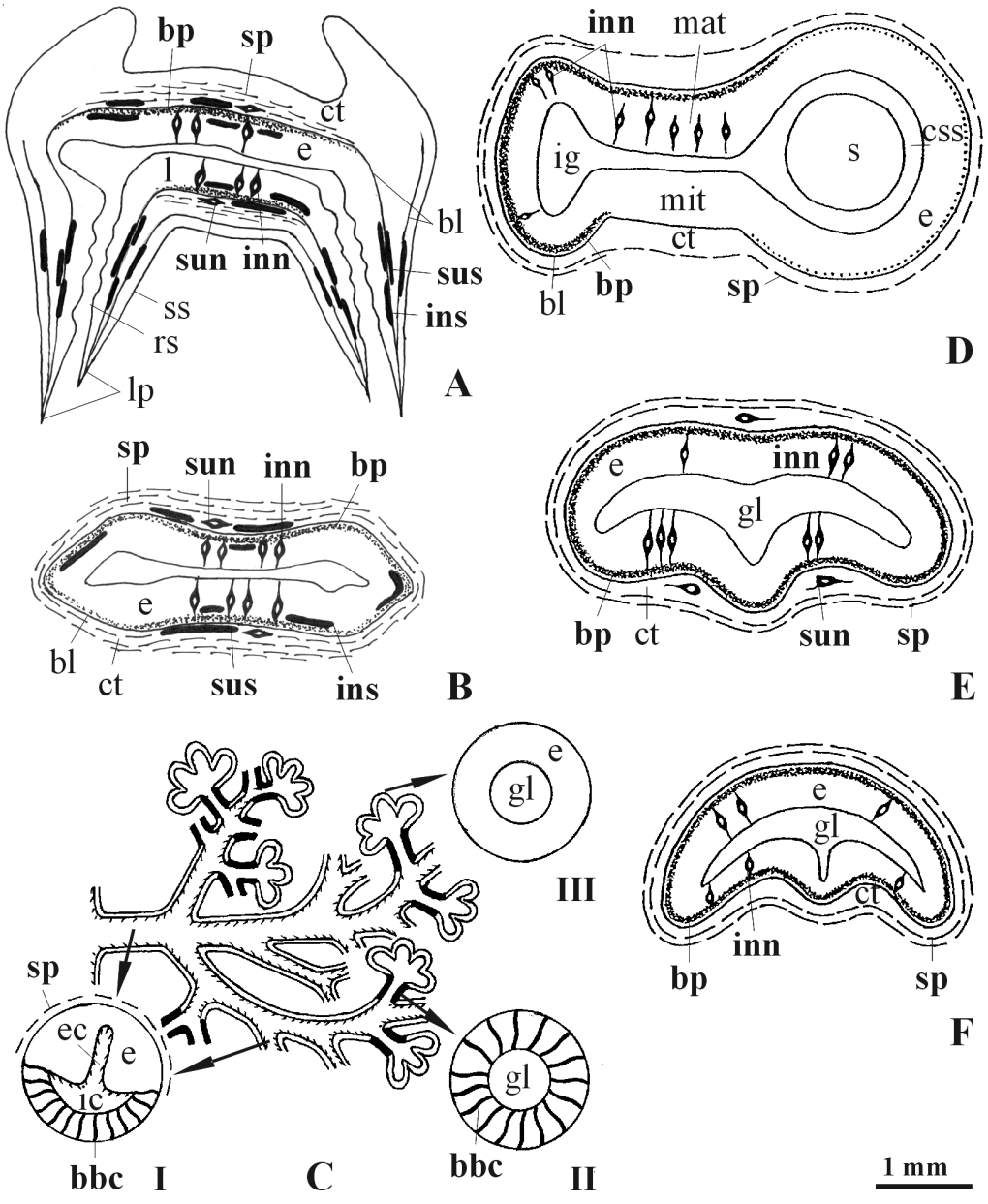

FIG. 1. Schematic drawing showing distribution of NADPH-d-positive elements in the digestive system of *Modiolus modiolus*. A: Labial palps and lips; B: Upper esophagus; C: Digestive gland; D: Anterior midgut; E: Midgut; F: Hindgut. Abbreviations: I, primary duct; II, secondary duct; III, digestive tubule; bbc, brush border cell; bl, basal lamina; bp, basiepithelial plexus; css, crystalline style sac; ct, connective tissue; e, epithelium; ec, excurrent canal; gl, gut lumen; ic, incurrent canal; ig, intestinal groove; inn, intraepithelial nerve cell; ins, intraepithelial secretory cell; l, lip; lp, labial palps; mat, major typhlosole; mit, minor typhlosole; rs, ridged side of the labial palp; s, crystalline style; sp, subepithelial plexus; ss, smooth side of the labial palp; sun, subepithelial nerve cell; sus, subepithelial secretory cell. Note: NADPH-d-positive elements are indicated by bold lettering.

TABLE 2. Major characters of NADPH-d-positive neurons and plexuses in various regions of the digestive tube of bivalve mollusks (Mytilidae); + indicates that rare cells or loose networks of fibers are labeled with diformazan granules. 0 indicates that no positive labeling is observed. Regions of the anterior midgut: CSS, crystalline style sac; IG, intestinal groove; MaT, major typhlosole; MiT, minor typhlosole. *data were previously published in Pimenova & Varaksin (2003); †data were previously published in Varaksin et al. (2002); ‡data were previously published in Pimenova et al. (2002).

Species		NADPH-d-positive cell proportion, %	Cell size, µm		Plexus thickness, µm	
			width	height	basi-epithelial	sub-epithelial
Lips						
*Crenomytilus grayanus**		0.30	3.9 ± 0.1	16.9 ± 0.3	7.9 ± 0.2	71.8 ± 1.4
Modiolus modiolus		0.24	4.7 ± 0.1	17.4 ± 0.3	13.4 ± 0.5	249.1 ± 2.9
Mytilus coruscus		0	0	0	3.7 ± 0.1	27.4 ± 0.5
Upper esophagus						
*Crenomytilus grayanus**		1.01	3.5 ± 0.1	15.1 ± 0.3	7.3 ± 0.2	75.9 ± 1.3
Modiolus modiolus		0.46	4.7 ± 0.1	16.3 ± 0.2	10.9 ± 0.2	181.5 ± 2.7
Mytilus coruscus		0	0	0	5.3 ± 0.2	33.9 ± 0.6
Middle esophagus						
*Crenomytilus grayanus**		0	0	0	+	36.7 ± 0.7
Modiolus modiolus		0	0	0	6.5 ± 0.2	151.3 ± 2.1
Mytilus coruscus		0	0	0	5.5 ± 0.2	41.9 ± 0.6
Lower esophagus						
*Crenomytilus grayanus**		0	0	0	+	33.9 ± 0.8
Modiolus modiolus		0	0	0	+	76.5 ± 1.4
Mytilus coruscus		0	0	0	+	32.1 ± 0.9
Anterior midgut						
Crenomytilus grayanus†	IG	0.51	3.6 ± 0.1	10.9 ±0.2	13.7 ± 0.5	30.1 ± 0.8
	MaT	1.28	5.7 ± 0.2	14.9 ± 0.3	6.7 ± 0.3	31.9 ± 1.1
	MiT	0	0	0	0	12.0 ± 0.7
	CSS	0	0	0	4.2 ± 0.1	5.2 ± 0.1
Modiolus modiolus‡	IG	0.17	2.9 ± 0.1	8.7 ± 0.2	7.5 ± 0.1	26.5 ± 0.3
	MaT	0.20	3.3 ± 0.1	10.8 ± 0.2	5.9 ± 0.2	+
	MiT	0	0	0	0	+
	CSS	0	0	0	8.3 ± 0.2	+
Mytilus coruscus	IG	+	3.9 ± 0.2	10.6 ± 0.3	10.5 ± 0.3	23.6 ± 0.7
	MaT	+	3.4 ± 0.2	13.9 ± 0.3	6.1 ± 0.2	20.7 ± 1.1
	MiT	0	0	0	+	+
	CSS	0	0	0	4.4 ± 0.1	+
Midgut						
Crenomytilus grayanus†		0.83	7.9 ± 0.1	17.3 ± 0.3	11.3 ± 0.2	28.7 ± 0.4
Modiolus modiolus‡		0.14	3.4 ± 0.1	15.5 ± 0.2	14.9 ± 0.1	37.3 ± 0.7
Mytilus coruscus		0	0	0	9.2 ± 0.4	16.1 ± 0.4
Hindgut						
Crenomytilus grayanus†		0.92	5.7 ± 0.1	17.9 ± 0.5	17.3 ± 0.4	66.8 ± 1.2
Modiolus modiolus‡		0.12	3.7 ± 0.1	11.9 ± 0.2	13.5 ± 0.3	57.1 ± 1.9
Mytilus coruscus		0	0	0	10.8 ± 0.3	14.9 ± 0.3

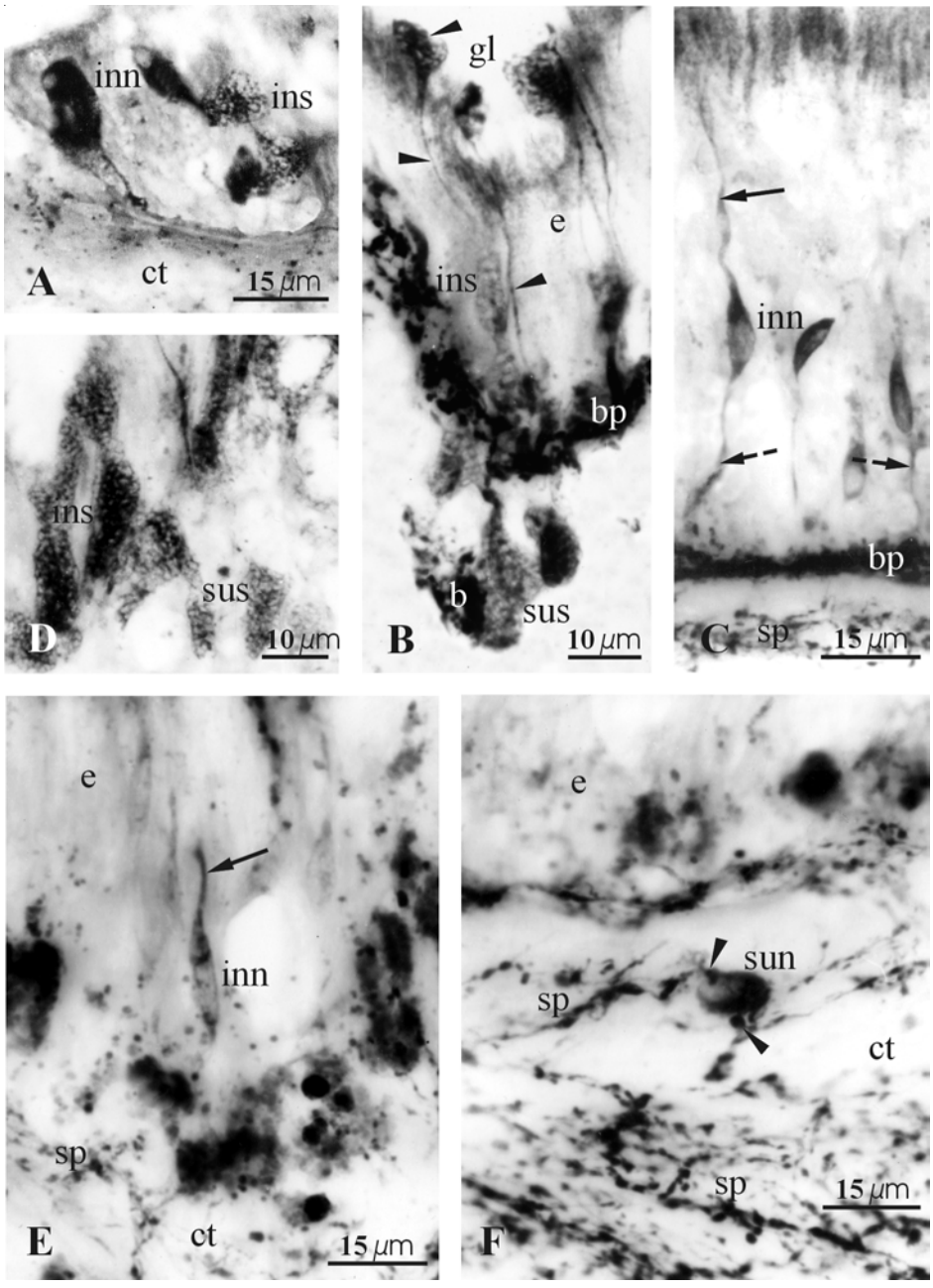

FIG. 2. NADPH-d-positive elements in the labial palps (A), lips (B, C) and upper esophagus (D, E, F) of bivalve mollusks. A: Intraepithelial nerve cells and secretory cell in *Crenomytilus grayanus*; B: Intraepithelial and subepithelial secretory cells in *Modiolus modiolus* (arrowheads show processes of subepithelial cells ending with terminal bulb); C: Intraepithelial nerve cells and plexuses in *Crenomytilus grayanus*; D: Intraepithelial and subepithelial secretory cells in *Mytilus coruscus*; E: Intraepithelial nerve cell in *M. modiolus*; F: Subepithelial nerve cell in *M. modiolus* (arrowhead indicate putative contacts between the neuron and fibers of the subepithelial plexus). Abbreviations: b, transversely cut bundle of nerve fibers; for the other abbreviations, see the caption to Fig. 1. Solid arrows show apical processes emerging from neuronal perikarya. Dashed arrows indicate basal neuronal processes.

Of the species examined, the most prominent basi- and subepithelial networks of nerve processes were observed in the lips of *M. modiolus* (Table 2). In the basal region of the epithelium, NADPH-d-histochemistry labeled longitudinal nerve fibers (Figs. 2B, C). A network of varicose fibers in the subjacent connective tissue also showed positive staining. In the dorsal and ventral regions of the lips, the labeling visualized subepithelial transverse fibers and thick longitudinal bundles of nerve processes (Figs. 2B, C). One could often see short neural bridges connecting the basiepithelial and subepithelial plexuses.

Esophagus

The slit-like mouth opens into a dorsoventrally flattened upper esophagus. The middle and lower parts of the esophagus pass through the digestive gland and are more regularly rounded in shape in cross section. The esophagus is a relatively short, hollow tube with longitudinal epithelial folds.

Upper Esophagus: NADPH-d-positive secretory cells were found in all the mytilid species examined. These cells were morphologically similar to the corresponding cell type of the lips and labial palps. They were most abundant in the dorsal and ventral regions of the upper esophagus (Fig. 1B) and lay both intraepithelially and beneath the epithelium (Fig. 2D). The intraepithelial cells were goblet-shaped, measuring 11.4 ± 0.2 µm in width and 21.6 ± 0.8 µm in height in *C. grayanus*. The nucleus was located close to the basal lamina, whereas the secretory process extended toward the gut lumen and ended with a terminal bulb. The subepithelial cells were more regularly rounded in shape.

NADPH-d-positive nerve cells occurred in the epithelium of *C. grayanus* and *M. modiolus*. They had an elongated fusiform cell body and a thin apical process that ran toward the gut lumen (Fig. 2E). These cells were most abundant in the basal part of the epithelium of the dorsal and ventral regions of the upper esophagus (Fig. 1B). In *C. grayanus*, these cells constituted 1.01% of the total number of epithelial cells (Table 2).

In all species studied, the esophageal epithelium possessed a basiepithelial plexus composed of tightly packed longitudinal NADPH-d-positive fibers (Figs. 2E, F) arranged parallel to one another and showed varicosities of varying shape and size. Of the bivalves studied, the plexus was most highly developed in *M. modiolus* (10.9 ± 0.2 µm thick) (Table 2). There was a direct connection between the basiepithelial plexus and the subepithelial plexus via thin neural bridges or solitary fibers. The subepithelial NADPH-d-positive plexus was composed of thin to thick transverse varicose fibers (Fig. 2F). Longitudinal bundles of densely packed fibers ran along the dorsal and ventral side of the upper esophagus. The subepithelial plexus was also most highly developed in *M. modiolus* (Table 2).

The subepithelial plexus of *M. modiolus* and *C. grayanus* contained rare cells that we regarded as putative neurons (Fig. 2F). These cells were subspherical to elliptical, with major and minor diameters averaging 7.6 ± 0.1 and 11.4 ± 0.2 µm in *M. modiolus* and 6.1 ± 0.1 and 11.7 ± 0.1 µm in *C. grayanus*, respectively. The cells were mostly unipolar, but some are bipolar. The perinuclear cytoplasm and proximal portions of the processes were homogeneously labeled with diformazan, but we failed to visualize the distal parts of the processes.

Middle Esophagus: In all mollusks studied, the epithelium of this portion of the digestive tract contained rare, weakly stained secretory cells. They occurred mostly in the lateral regions, lying either separately or in groups of two to three. No NADPH-d-positive neurons were found.

Basi- and subepithelial NADPH-d-positive plexuses were found in all the mytilid species examined, but also were most highly developed in *M. modiolus* (Table 2). The basiepithelial plexus of the middle esophagus consisted of mostly longitudinally arranged NADPH-d-positive fibers (Figs. 3A, B). NADPH-diaphorase histochemistry also labeled numerous longitudinal, transverse, and oblique fibers of varying diameter and staining intensity that constituted the subepithelial plexus (Figs. 3A, B). The connective tissue that underlay the dorsal and ventral regions of the digestive epithelium often contained longitudinal bundles of nerve processes (Fig. 3A). The two plexuses were connected via solitary fibers (Fig. 3B). In *C. grayanus* and *M. modiolus*, diformazan also labeled the perikarya and processes of subepithelial neurons. These were small unipolar cells with a subspherical to elliptical cell body (Fig. 3C) with major and minor diameters of 7.9 ± 0.1 and 12.9 ± 0.2 µm in *M. modiolus* and 6.5 ± 0.1 and 10.2 ± 0.1 µm in *C. grayanus*, respectively.

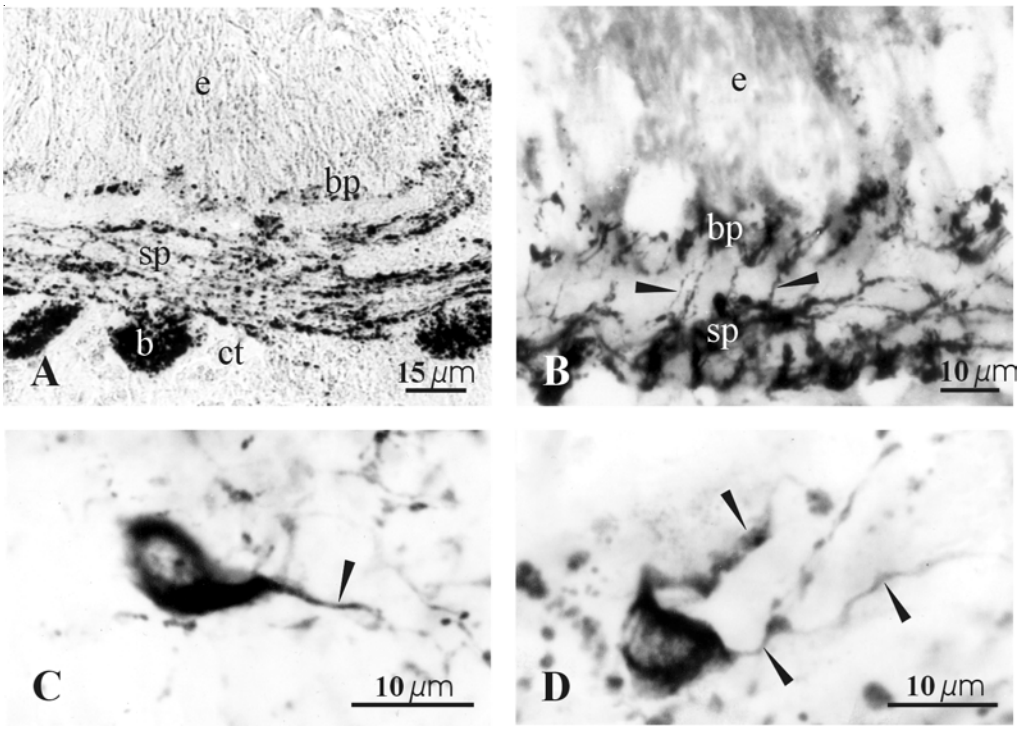

FIG. 3. NADPH-d-positive elements in the middle (A, B, C) and lower (D) esophagus of bivalve mollusks. A: Basiepithelial and subepithelial plexuses in *Crenomytilus grayanus*; B: Connection between the basi- and subepithelial plexuses via solitary fibers (arrowheads) in *Mytilus coruscus*; C: Subepithelial unipolar (arrowhead) neuron in *Modiolus modiolus*; D: Subepithelial bipolar (arrowheads) neuron in *M. modiolus*. Abbreviations are the same as in Figs. 1 and 2.

Lower Esophagus: NADPH-d-positive secretory cells, either scattered singly or clustered together to form small groups, occurred in the lateral regions of the digestive epithelium. Positively stained nerve cells were absent.

The basi- and subepithelial plexuses were composed of fibers of varying diameter and staining intensity (Table 2). The dorsal and ventral regions of the digestive epithelium were underlain by connective tissue that contained thick longitudinal bundles of nerve processes. The two plexuses were interconnected via solitary fibers.

Singly-scattered subepithelial NADPH-d-positive nerve cells occurred in the lower esophagus of *C. grayanus* and *M. modiolus*. These cells had an elliptical cell body that gave rise to one to two processes (Fig. 3D). The perinuclear cytoplasm and processes showed clear positive labeling, while the nucleus remained unstained. The major and minor diameters of the perikarya ranged from 7.8 ± 0.2 and 11.6 ± 0.1 μm (in *M. modiolus*) to 10.2 ± 0.1 and 6.5 ± 0.1 μm (in *C. grayanus*), respectively.

Stomach

The stomach is a sac-shaped organ lined with a heavily ridged mucosa. It is anteriodorsally continuous with the esophagus and leads posterioventrally to the intestine. The stomach is surrounded by the digestive gland, the ducts of which open into the stomach lumen. The anterior portion of the stomach includes a large sorting area with gastric grooves and ridges, and a gastric shield, which is covered by an extracellular plate. The posterior stomach contains a typhlosoles, crystalline style sac, and an intestinal groove.

In all species studied, the heaviest staining was observed in the gastric grooves of the sorting area (Figs. 4A, B). The NADPH-d-positive plexus lay in the basal region of the intes-

tinal groove and consisted of a network of thin longitudinal nerve processes. Singly scattered fibers also ran along either side of the groove. The underlying connective tissue contained rare thin fibers that gave off no branches and ran parallel to the basal surface of the epithelium. Only in *M. coruscus*, NADPH-d-histochemistry labeled rare intraepithelial nerve cells in the basal part of the gastric grooves (Fig. 4B). These cells measured 3.5 ± 0.1 µm in width and 14.1 ± 0.3 µm in height. They either rode upon the basiepithelial plexus or were connected to the latter via their basal processes. The apical process extended toward the gut lumen.

The ridges of the sorting area had no intraepithelial NADPH-d-positive cells and basiepithelial plexus. The underlying connective tissue contained longitudinal bundles of nerve fibers, which, in transverse sections, appeared as either singly-scattered dots or patches of varying morphology and staining intensity.

No NADPH-d-positive elements were found in the gastric shield region. Rare varicose fibers occurred only in the underlying connective tissue.

Of the species examined, only *C. grayanus* possessed intraepithelial nerve cells in the typhlosoles of the posterior stomach (Fig. 4C). They were relatively numerous, and had an elongated fusiform perikaryon and two processes that arose from the opposite poles of the cell body. One of these processes ascended toward the gut lumen, whereas another contacted a moderately developed NADPH-d-positive basiepithelial plexus. These were 3.5 ± 0.03 µm wide and 27.2 ± 0.3 µm high.

In the crystalline style sac and intestinal groove of the posterior stomach of the mytilids, NADPH-d-histochemistry labeled only the basi- and subepithelial plexuses.

Digestive Gland

The digestive gland consists of large primary ducts that extend from the stomach wall and give off short undivided secondary ducts. The latter are continuous with digestive tubules (Fig. 1C).

The lumen of the primary ducts is provided with typhlosole-like longitudinal ridges that serve to separate it into two canals (Fig. 1Cl). One the latter is lined with brush border cells and fulfills the functions of an incurrent canal that transports food particles from the stomach to the digestive tubules. The remainder of the lumen functions as an excurrent canal, through which the undigested particles and dead epithelial cells of the tubules pass to the stomach.

In the bivalve species examined in this study, the brush border epithelial cells of the incurrent canal showed homogeneous positive labeling with diformazan (Fig. 4D). These ribbon-shaped cells extended the whole height of the epithelium from the basal lamina to the apical surface and terminated with a small apical bulb (Fig. 4E). The underlying connective tissue contained singly scattered NADPH-d-positive fibers.

In all mytilids, the ciliated epithelial cells of the excurrent canal showed no staining for NADPH-diaphorase (Fig. 4D). NADPH-d-histochemistry labeled a loose subepithelial plexus composed of poorly arborized thin nerve fibers (Fig. 4D).

The secondary ducts were lined with brush border cells (Fig. 1Cll), which, like the cells of the incurrent canal, showed positive labeling for NADPH-diaphorase (Fig. 4F). Rare, thin NADPH-d-positive fibers with small varicosities occurred only beneath the epithelium. They were poorly arborized and extended deeply into the underlying connective tissue.

In all species studied, the cells of the digestive tubules exhibited no positive labeling for NADPH-diaphorase (Figs. 1Clll, 4F). The underlying connective tissue contained numerous fibers with heteromorphic varicosities. These fibers were either scattered singly or arranged in thick bundles (Figs. 4D, F).

Intestine

The intestine is conventionally subdivided into the anterior midgut that contains a crystalline style, the midgut proper, and the hindgut.

Anterior Midgut: In mytilids, the anterior midgut is composed of a tube that possesses the intestinal groove, the major and minor typhlosoles, and the crystalline style sac (Fig. 1D). In all mollusks studied, the most intense NADPH-d labeling was observed in the intestinal groove (Fig. 5A). The subpopulation of NAPDH-d-positive intraepithelial neurons ranged from a single cell (in *M. coruscus*) to 0.51% of the total number of epithelial cells (in *C. grayanus*) (Table 2). A thin apical process extended toward the gut lumen. The basal region of the intraepithelial neurons was

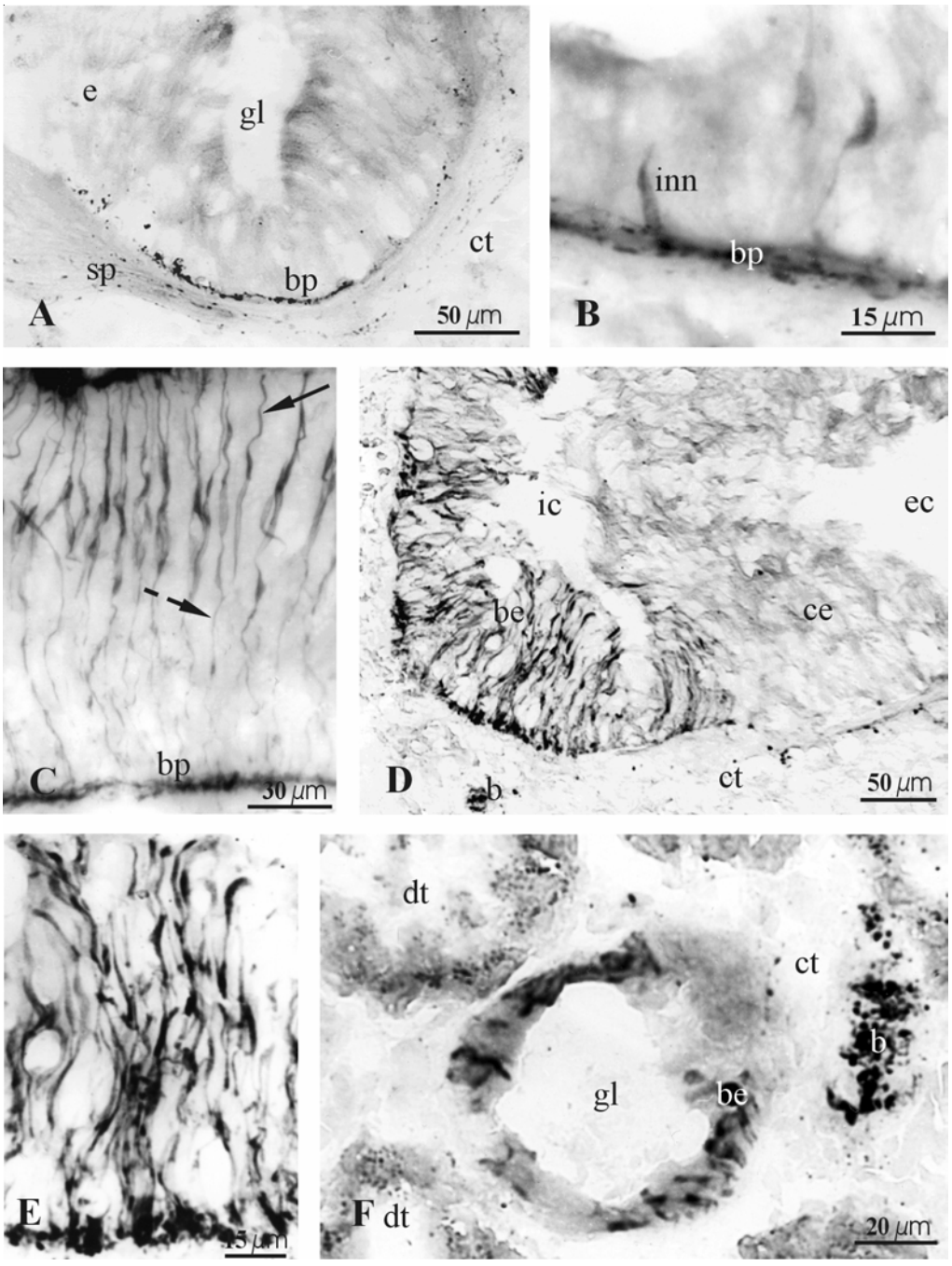

FIG. 4. NADPH-d-positive elements in the stomach (A, B, C) and digestive gland (D, E, F) of bivalve mollusks. A: Plexuses in the gastric groove of *Crenomytilus grayanus*; B: Nerve cells in the epithelium of the gastric groove in *Mytilus coruscus*; C: Nerve cells in the epithelium of the typhlosole in *C. grayanus*; D: Primary duct of the digestive gland in *C. grayanus*; E: Brush border epithelial cells of the primary duct in *C. grayanus*; F: Secondary duct of the digestive gland in *C. grayanus*. Abbreviations: be, brush border epithelium; ce, ciliated epithelium; dt, digestive tubule. See caption to Figs. 1 and 2 for the remaining abbreviations.

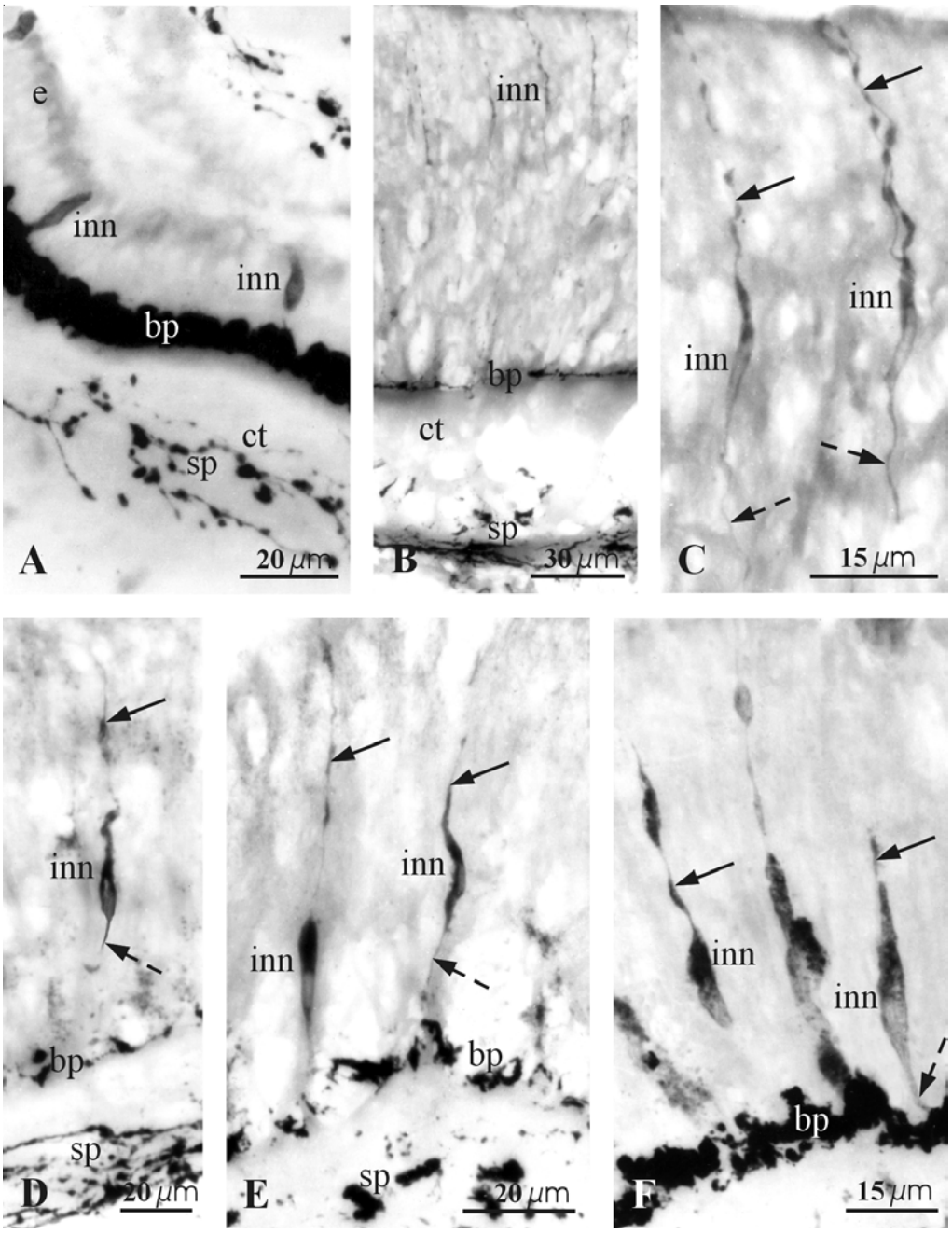

FIG. 5. NADPH-d-positive elements in the intestine of the bivalve mollusks. A: Intraepithelial nerve cells and plexuses in the intestinal groove of *Crenomytilus grayanus*; B: Major typhlosole in the anterior midgut of *C. grayanus*; C: Nerve cells in the epithelium of the major typhlosole in *C. grayanus*; D: Midgut of *Modiolus modiolus*; E: Hindgut of *M. modiolus*; F: Nerve cells and plexus in the epithelium of the hindgut in *C. grayanus*. See caption to Figs. 1 and 2 for abbreviations.

swollen and contributed to the basiepithelial plexus. The latter showed an intense blue staining and was composed of numerous bundles of nerve fibers. The NADPH-d-positive basi- and subepithelial plexuses were interconnected via thin fibers.

The epithelium of the major typhlosole also contained NADPH-d-positive nerve cells, which possessed a fusiform cell body, and were interspersed among other cell type in the apical region of the epithelium (Figs. 5B, C). These cells also occasionally occurred between the intestinal groove and major typhlosole and between the major typhlosole and crystalline style sac. The labeled cells were most abundant (1.28%) in the epithelium of *C. grayanus* (Table 2). In the major typhlosole of the all species studied, the basal region of the epithelium showed a dense network of longitudinal positively stained fibers (Fig. 5B). NADPH-d-histochemistry also labeled cross cut bundles of nerve fibers that lay in the segment, adjacent to the crystalline style sac. The basiepithelial plexus was connected with the NADPH-d-positive subepithelial plexus (Fig. 5B).

No positive labeling for NADPH-diaphorase was observed in the cells of the minor typhlosole. The basiepithelial plexus was also often absent (Fig. 1D); however, rare fibers occurred in the basal portion of the epithelium only in *M. coruscus*. The subepithelial NADPH-d-positive varicose fibers were arranged in loose networks (Table 2).

The epithelium of the crystalline style sac also showed no positive staining for NADPH-diaphorase. The basi- and subepithelial plexuses were composed of loosely spaced fibers (Table 2; Fig. 1D).

Midgut: NADPH-d-positive neurons were found in the gut epithelium of *C. grayanus* and *M. modiolus* (Figs. 1E; 5D). The thin apical process was clearly seen; it ran from the cell body toward the gut lumen. The basal process contributed to the basiepithelial plexus. The intraepithelial neurons constituted 0.14% (in *M. modiolus*) to 0.83% (in *C. grayanus*) of the total number of epithelial cells.

The NADPH-d-positive basiepithelial plexus was composed of longitudinally oriented fibers (Fig. 5D). As in other gut regions, the basi- and subepithelial plexuses were loosely connected via solitary nerve fibers. The subepithelial plexus consisted of densely packed fibers, showed a dark-blue staining (Fig. 5D), and measured 16.1 ± 0.4 µm (in *M. coruscus*) to 37.3 ± 0.7 µm (in *M. modiolus*) thick (Table 2). Only in *M. modiolus*, on the ventral (concave) side of the intestine and occasionally on the dorsal (convex) side, the subepithelial plexus contained NADPH-d-positive nerve cells (Fig. 1E), with the minor and major diameters of 5.2 ± 0.2 and 13.5 ± 0.2 µm, respectively. These were most often unipolar neurons; however, some were bipolar, and the least abundant were multipolar neurons. Their processes either contributed to the subepithelial plexus or extended deeply into the connective tissue.

Hindgut: The hindgut is a hollow tube that, after entering the digestive gland, and passes through the pericardial sac and the ventricle of the heart, proceeds to the posterior adductor muscle, passes over the dorsal surface of the latter to end at the anus.

In the hindgut of *C. grayanus* and *M. modiolus*, NADPH-d-histochemistry stained intraepithelial nerve cells (Table 2; Fig. 1F). These were fusiform cells that were either scattered singly or clustered together to form small groups (Figs. 5E, F). Diformazan homogeneously labeled the perikarya and the processes to their full extent, including the most distal parts, while the nucleus remained unstained.

TABLE 3. Distribution of NADPH-d-positive elements in the digestive system of bivalve mollusks (Mytilidae); ++, intraepithelial NADPH-d-positive neurons and plexuses; +, NADPH-d-positive plexuses; 0, no NADPH-d-positive neurons and plexuses were observed; (+), NADPH-d-positive secretory cells; [+], NADPH-d-positive brush border cells.

Species	Labial palps	Lips, upper esophagus	Middle and lower esophagus	Stomach	Digestive gland	Midgut	Hindgut
Crenomytilus grayanus	0 (+)	++ (+)	+ (+)	++	+ [+]	++	++
Modiolus modiolus	+ (+)	++ (+)	+ (+)	+	+ [+]	++	++
Mytilus coruscus	0 (+)	+ (+)	+ (+)	++	+ [+]	++	+

In all species studied, the NADPH-d-positive basiepithelial plexus consisted of a dense heavily stained network of nerve fibers. The subepithelial plexus comprised varicose fibers that ran in various directions and were often arranged in bundles (Figs. 5E, F).

The data presented above suggest that NADPH-d-positive elements occur in all parts of the bivalve digestive system and include heteromorphic cells and nerve plexuses (Table 3).

No positive labeling for NADPH-diaphorase was observed in the control sections.

DISCUSSION

Nerve Cells and Plexuses

The paper gives an account of distribution and morphology of putative nitroxidergic cells and fibers in the digestive system of *Crenomytilus grayanus*, *Modiolus modiolus*, and *Mytilus coruscus* (Mytilidae). In the bivalves studied, virtually all of the gut regions contain NADPH-d-positive fusiform cells, the thin apical process of which extends toward the gut lumen, while the basal process runs in the opposite direction and feeds into the basiepithelial putative nitroxidergic plexus. In the gut epithelium of many invertebrate taxa, including bivalves, specialized cells were found that are not directly involved in digestion but fulfill regulatory functions. In bivalves, the enteric regulatory system is well developed and seems to comprise both nerve and endocrine cells (Punin, 2001). Electron microscopy studies of the intestinal epithelium in *Arctica islandica* and *Mytilus edulis* revealed cells that were similar to neurons in terms of ultrastructural features (Punin, 1981, 1989). These cells possess a well-developed, rough endoplasmic reticulum, prominent Golgi bodies, various types of granules, and processes that arise from the perikaryon and feed into the basiepithelial plexus. Vital staining with methylene blue visualizes receptor nerve cells in the midgut epithelium of *Anodonta cellensis* (Gilev, 1952). Our results show that the intraepithelial cells in the lips, esophagus, stomach, and intestine of the bivalves examined are similar, both in terms of morphology and distribution, to the cells of the enteric regulatory system of the bivalves studied by other authors (Punin, 1981, 1989, 2001; Gilev, 1952). These cells seem to meet all morphological criteria of nerve cells.

According to reports of a number of authors, the lips, mouth, tentacles, and pneumostome of gastropod mollusks are provided with aggregations of neurosensory cells that give rise to sensory organs in some species (Zylstra, 1972). For instance, receptor cells were found beneath the epidermis of the foot, head, mouth, lips, and tentacles in the pulmonates *Lymnaea stagnalis* and *Helix vulgaris* (Zaitseva, 1980). The author refers to these cells as primary bipolar receptors.

Our results demonstrate that in bivalves studied, virtually all regions of the digestive system contain fibers that stain blue with NADPH-d-histochemistry and lie both in the basal region of the epithelium and in the underlying connective tissue. It has been previously shown that the alimentary canal of bivalves is provided with a complex system of basiepithelial and subepithelial nerve plexuses (Giusti, 1970; Punin, 1981, 1989). Electron microscopy examination shows that the two plexuses differ in size of granules in the nerve processes and, therefore, may contain different biologically active substances. The subepithelial plexus is thought to be a part of the peripheral nervous system (Punin & Konstantinova, 1988; Punin, 1989).

Vital staining with methylene blue and histofluorescence studies suggest that the basiepithelial plexus is composed of processes that arise from the intraepithelial cells (Punin, 1981; Punin & Konstantinova, 1988). However, if we assume that only intraepithelial NO-ergic cells send their nerve processes to the basiepithelial NO-ergic plexus, then it would be difficult to explain why the latter is so well developed in certain cases. This plexus is most probably connected with cells and fibers that lie in the connective tissue. Light and electron microscopy studies on *M. edulis* (Punin & Konstantinova, 1988) and *A. islandica* (Punin, 1981), respectively, demonstrate that nerve processes of the basiepithelial nerve plexus occasionally penetrate into the underlying connective tissue. Moreover, in the hindgut of *Tapes watlingi* (Dougan & McLean, 1970) and midgut of *Mytilus galloprovincialis* (Giusti, 1970), nerve fibers lying in the connective tissue were found to invade the basal region of the epithelium. Consistent with these observations, we often observed nitroxidergic fibers that connected the NO-ergic, basi- and subepithelial plexuses. The above findings suggest that the two plexuses should be referred to as interconnected parts of the anatomically continuous bivalve enteric nervous system.

It is worth noting that in spite of a relatively small proportion of putative NO-ergic cells in the gut epithelium, the basiepithelial plexus is highly developed. We have elsewhere shown that although only relatively a small number of perikarya show positive labeling in ganglia of bivalve mollusks, the neuropile areas contain plenty of NO-ergic fibers (Annikova et al., 2000). A similar pattern of staining was observed in the central nervous system of other bivalve species, as well as in gastropods (Moroz & Gillette, 1996; Dyuizen et al., 1999). A study carried out on the bivalve *C. grayanus* suggests that NO production is not restricted to the cell body; nerve processes are believed to be capable of local synthesis of nitric oxide (Annikova et al., 2000). Consistent with this suggestion, our results showed the localization of NO-synthase in numerous fibers that bear varicosities along their length. Varicose swellings have been commonly regarded as sites where neurotransmitters and other biologically active substances are produced and released.

In *C. grayanus* and *M. modiolus*, the subepithelial plexus contained singly scattered unipolar, bipolar or sometimes multipolar NADPH-d-positive cells. In the esophagus of *A. islandica* and intestine of *M. edulis*, the subepithelial tissues contain a cell type with processes that penetrate into the epithelium to extend toward the gut lumen (Punin, 1981, 2001; Punin & Konstantinova, 1988). Our results showed that the processes of subepithelial NO-ergic cells either terminated within the adjacent tissues or contacted the fibers of the subepithelial plexus rather than invading the epithelium. Gilev (1952) visualized bipolar neurons in the subepithelial plexus of *A. cellensis* using methylene blue staining. The author referred to these cells as receptors generating sensory impulses. In pulmonates, rare nerve cells were found in the subcutaneous plexus and among the distal branches of nerves in the head, mouth, lips, and tentacles (Zaitseva, 1980). Most of these cells are believed to be interneurons that mediate horizontal and vertical connections between receptors and are responsible for interactions between the sensory cells and efferent fibers. These data are consistent with our hypothesis that putative NO-ergic cells in the digestive system of bivalves are nerve cells that are probably involved in transmission of sensory signals to the central nervous system and in preliminary processing of information.

Thus, our results show that the digestive system of bivalve mollusks is provided with a well-developed putative nitroxidergic nervous system, which comprises intra- and subepithelial neurons, basi- and subepithelial plexuses. Putative nitroxidergic cells can act both as receptors and as effectors and, therefore, can be regarded as components of local reflex arcs. The NO-ergic cells seem to be connected to the central nervous system and thereby fulfill functions of a sensory component of regulatory circuits (Balashov et al., 1992; Punin, 2001). These putative receptors might release a biologically active substance in response to chemical stimuli from the gut lumen. The intestinal epithelium of bivalves is known to possess a complex heterogeneous system of regulatory cells, which contain various monoamines and oligopeptides (Punin, 2001). Our studies have shown that, besides other biologically active substances, these cells seem to produce nitric oxide.

Nitric oxide is believed to play a key role in sensory processing mechanisms (particularly, in the olfactory system) in various animal taxa ranging from coelenterates to mammals (Colasanti & Venturini, 1998). Studied carried out on gastropods, the nitroxidergic neurons of which have been extensively studied, also suggest that these cells primarily act as receptors (Moroz et al., 2000; Gelperin et al., 2000, 2001).

Secretory and Brush Border Epithelial Cells

Besides putative nitroxidergic nerve cells and fibers, NADPH-d-histochemistry also labels non-neuronal cells in the anterior regions of the digestive system and in the digestive gland of the species studied. These cells differ from the putative NO-ergic neurons both in morphology and distribution pattern.

In the labial palps, lips, and esophagus, we observed numerous intra- and subepithelial secretory cells that stained positive for NADPH-diaphorase. Frolova (1988) previously showed that, besides predominant ciliated cells, numerous intra- and subepithelial secretory cells occur in the labial pulps, lips, and esophagus of *C. grayanus*. This suggests that in the labial palps, lips, and esophagus of the species studied some of the intra- and subepithelial secretory cells do contain NO-synthase.

NADPH-diaphorase labeling was also observed in brush border cells, but not in ciliated

cells, in the luminal epithelium of the primary and secondary ducts of the digestive gland. This labeling seems to be due to the presence of NO-synthase in the brush border cells that line the incurrent canal of the primary and secondary ducts, the major function of which is to transport food particles (Leibson & Usheva, 1979).

NO-synthase was found both in neurons and non-neuronal cell types, including epithelial cells, in a variety of animal taxa and in human tissues (Proskuryakov et al., 1999). For instance, NADPH-d-histochemistry labeled non-neuronal cells in the peripheral olfactory organs of the gastropod *Clione limacina* (Moroz et al., 2000). The authors referred to them as secretory-like cells. In another study, circumoral epithelial tissues of the nudibranch *Melibe leonina* showed positive staining for NO-synthase (Newcomb & Watson, 2001). In the earthworm *Lumbricus terrestris*, NO-synthase immunoreactivity was seen in the mucous cells within the epidermis (Licata et al., 2002).

Possible Functional Significance of Nitric Oxide

Heavy NADPH-diaphorase staining labels the intraepithelial neurons and basiepithelial plexus that make direct contacts with ciliated epithelial cells of the digestive tube. Nitric oxide is believed to be capable of controlling ciliary beating frequency, as was shown for the epithelial cells of the mantle integument of the mussel *M. galloprovincialis* (Licata et al., 2003). Both our data and reports of other authors suggest that nitric oxide regulates ciliary currents that transport food particles and feces along the digestive tube. However, no NO-ergic cells were observed associating with the epithelium of the crystalline style sac, which possessed a well-developed locomotory apparatus. Surprisingly, the basiepithelial plexus is not highly elaborated in this epithelium. This suggests that nitric oxide is not the only substance that affects the ciliary beating in the digestive tract of bivalve mollusks. Similarly, NADPH-d labeling is not evenly distributed throughout the ducts of the digestive gland in the species studied. It is worth noting that only rare positively stained subepithelial fibers occur beneath the ciliated epithelium of the excurrent canal of the primary duct, whereas almost a half of brush border epithelial cells of the incurrent portion of the primary and secondary ducts show specific staining with diformazan granules.

In addition to their main locomotory function, ciliated epithelial cells are also involved in secretion (mucous synthesis, secretion of digestive enzymes and proteinaceous components of the crystalline style), absorption, and deposition of nutrients (Owen, 1966; Reid, 1966; Giusti, 1970). This mostly applies to the cells of the epithelial lining of the lips, esophagus, intestinal groove, major typhlosole, midgut, and hindgut. These regions are provided with NO-ergic neurons and show well-developed basi- and subepithelial plexuses. On the other hand, the minor typhlosole and crystalline style sac have a relatively low secretory activity and, correspondingly, possess poorly developed nitroxidergic plexuses and no nitroxidergic cells. This suggests that nitric oxide is involved in the regulation of secretion and absorption in the gut of bivalve mollusks. This is consistent with our observations of numerous NADPH-d-positive secretory cells in the anterior regions of the digestive system. The role of nitric oxide in the control of secretion and absorption in the mammalian alimentary canal has been extensively studied (Schleiffer & Raul, 1997; Shah et al., 2004). It has been also demonstrated that NO is involved in the regulation of mucous secretion in the mantle integument of *M. galloprovincialis* and epidermis of *L. terrestris* (Licata et al., 2002, 2003).

Thus, our data show that the digestive system of the mytilids *Crenomytilus grayanus*, *Modiolus modiolus*, and *Mytilus coruscus* contains a system of heteromorphous putative NO-ergic cells. NADPH-diaphorase was demonstrated to occur in the intra- and subepithelial nerve cell, as well as in the basi- and subepithelial nerve plexuses in all regions of the digestive tube. Moreover, NADPH-diaphorase was detected in the intra- and subepithelial secretory cells of the labial palps, lips, and esophagus and in the brush border epithelial cells of the primary and secondary ducts of the digestive gland.

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